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A HPLC method to monitor the occurrence of lipid peroxidation in intravenous lipid emulsions used in parenteral nutrition using in-line UV and Charged Aerosol Detection.

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Abstract

Parenteral Nutrition (PN) provides life sustaining support where gastrointestinal nutrition is inadequate due to disease or prematurity. Intravenous lipid emulsions (IVLE's) form a staple part of PN. Whilst the physical stability of IVLE's is relatively well known and quantified, chemical stability is an area where little testing has occurred. We report a new sensitive method for the monitoring of selected triglycerides present within two IVLE's and the detection and quantification of the peroxidation product 4-hydroxynonenal (HNE) using HPLC with in-line UV and charged aerosol detection (CAD). IVLE's used included the soy-bean oil-based emulsion Intralipid® 20 % and SMOFlipid® 20 % (Fresenius Kabi UK), based on soy-bean, olive, fish oil and medium chain triglycerides. Assay validation gave R^2 values of ≥ 0.99 for all selected triglyceride peaks and 4-hydroxynonenal. Inter and intra-day repeatability gave RSD values < 7.2 % for CAD detection, achieving a precise and repeatable method. HNE was confirmed through internal standardisation of the HPLC method. Selected triglycerides were identified using ESI-MS with MicroTOF. This novel method permits the chemical stability of IVLE's to be quantified and monitored in respect to lipid peroxidation during storage prior to delivery to the patient, influencing the optimal safety conditions of IVLE's in a clinical setting.

Key words:

Methods, HPLC, Lipid Peroxidation, Lipids, Emulsions, Triglycerides, Lipids, Oxidised lipids, quantitation, Fatty acid oxidation, Non-aqueous reversed phase high performance liquid chromatography, Parenteral nutrition, Intravenous lipid emulsions.

Abbreviations: TAG, triglyceride; CAD, charged aerosol detector; NARP, non-aqueous reversed phase; IVLE, intravenous lipid emulsion; PN, parenteral nutrition; HNE, 4-hydroxynonenal; AIO, all-in-one; MDA, malondialdehyde; HHE, 4-hydroxyhexenal; HUE, 4-hydroxyundecenal; FOX ferrous oxidation-xynol orange; TBARS, Thiobarbituric Acid Reactive Substances; IPA, Isopropanol; ACN, Acetonitrile; LLOD, lower limit of detection; LLOQ, lower limit of quantification; SmPC, Summary of Product Characteristics; RSD, relative standard deviation; FK, Fresenius Kabi; MeOH, methanol.

Introduction

Intravenous lipid emulsions (IVLE's) form a staple part of parenteral nutrition (PN). PN provides life sustaining support where gastrointestinal nutrition is either not possible, or inadequate due to disease or prematurity. IVLE's are provided in PN either in combination all-in-one (AIO) mixtures with all other PN components (glucose, amino acids, vitamins, trace elements and electrolytes), or separated, in the case of delivery of PN to neonates [1]. Physical stability of PN is well studied and currently governs the combinations and concentrations of components permissible within a PN admixture for a patient. Lipid globule size, pH, visual inspection for signs of emulsion instability and globule size distribution are commonly tested to confirm physical stability [2–4]. Chemical stability of PN during storage after manufacturing is currently not extensively studied, but is an area which needs to be addressed due to the growing body of knowledge on the harmful effects of breakdown products of lipids due to peroxidation [5–9].

All unsaturated lipids are susceptible to peroxidation, a cyclical process resulting in the production of primary and secondary toxic peroxidation products [7,10]. Primary hydroperoxides are transient making their quantification a challenge. Therefore, secondary peroxidation products are more extensively tested. These include the tertiary peroxidation product malondialdehyde (MDA) and the secondary aldehydic products 4-hydroxynonenal (HNE), 4-hydroxyhexanal (HHE) and 4-hydroxundecenal (HUE) which are by far the most extensively studied with regards to their in vitro toxicity [5,11–16].

With regards to PN, Intralipid® 20% (Fresenius Kabi) has been the most extensively used IVLE and is formulated from soy bean oil rich in omega 6 linoleic acid, omega 9 oleic acid and omega 3 α -linolenic acid, proportions of each displayed in table 1 [17]. All contain levels of unsaturation and therefore are susceptible to peroxidation into secondary aldehydes HNE, HUE and HHE respectively. SMOFlipid® 20% (FK), a newer generation IVLE contains a mixture of soy bean, olive and fish oils and saturated

medium chain triglycerides as shown in table 1. Whilst the level of saturated fatty acids is higher in SMOFlipid[®] than Intralipid[®], both contain linoleic, oleic and α -linolenic acids that can peroxidise to the aldehydes mentioned. In an effort to inhibit peroxidation from occurring, SMOFlipid[®], which contains the longer polyunsaturated EPA and DHA, contains tocopherols which are proposed potent free radical scavengers [18]. In a clinical setting PN formulations are prepared often on an individual patient basis, manufactured in a hospital pharmacy setting. Formulations may be exposed to air during preparation and are stored in either oxygen permeable syringes for neonatal lipid PN or oxygen impermeable PN bags. Every effort is made to remove as much air as possible after manufacturing, but a level of oxygen remains providing the initial environment for peroxidation to occur.

Current assays available to quantify peroxidation include the TBARs assay [19,20], FOX assay [21] and HPLC assays looking for derivatised aldehydic products of peroxidation [22–24]. Whilst effective in certain conditions both the TBARs and FOX assays are unsuitable as are proven to be relatively inaccurate in their estimations of lipid peroxidation [21]. HPLC with derivatisation for the detection of HNE [25,26] is accurate and quantifiable but the derivatisation is time consuming and unnecessary as free HNE should theoretically be formed within IVLE's. HPLC with UV detection of free HNE has been documented and used successfully by Emerit et al. and Esterbauer et al. [27,28]. Here we present a method that provides accurate detection and monitoring of the triglycerides present within each IVLE via HPLC with charged aerosol detection (CAD) in-line with the UV detection of HNE, aiming to circumvent the limitations with current methods and complement the detection of HNE with a secondary method to monitor the breakdown of lipids occurring.

Materials and Methods

Reagents and Materials:

LC-MS grade isopropanol (IPA) for HPLC analysis, water with 0.1% formic acid, and methanol (MeOH) with 0.1% formic acid for MS analysis were purchased from Fisher Scientific. LC-MS grade acetonitrile for HPLC analysis was purchased from VWR chemicals. 4-Hydroxynonenal standard ($1 \text{ mg } 100 \mu\text{l}^{-1}$) was purchased from Santa-Cruz Biotechnology and used for HPLC method development. Intralipid® 20% (Batch numbers: 100 ml bags 10LH3938, 10LH3939, 10LI4439, EXP 07/19, 08/19, 08/19) and SMOFlipid® 20% IVLE's (500 ml bags batch numbers 10MA9062 EXP:12/19 and 250 ml bags batch number 10LG3032 EXP: 06/19) were kindly donated by Fresenius Kabi UK.

HPLC-UV-CAD conditions:

The analysis of the triglycerides and HNE was performed on a Thermo Scientific Ultimate 3000 HPLC system coupled firstly to a Spectra system UV detector and then to an in-line CAD. Gonyon et al. [29] provides an initial set of chromatographic conditions employing non-aqueous reversed phase HPLC from which the assay was developed. Separations were carried out on an Acclaim C30 column 3.0 mm x 250 mm, 3 μm particle size at a flow rate of 0.2 ml min^{-1} . The non-aqueous mobile phase consisted of an IPA (phase A) and ACN (phase B) gradient. The gradient program started at 60% phase B, decreased to 40% B over the first 20 minutes, was held at 40% B for a further 50 minutes then increased to 60% B over 15 minutes with a 5-minute isocratic hold for re-equilibration at the end of each run. The column temperature was held at 5°C after optimisation to maximise resolution and the autosampler was held at 8°C throughout to maintain refrigerated conditions of samples and inhibit further peroxidation from occurring during analysis. Injection volume was 1 μl of lipid and the sample loop was washed with 60 % MeOH/water after each injection. Sample preparation was unnecessary due to the ability of the CAD detector to detect triglycerides present within an emulsion without any sample extraction required [29]. The UV detector was set and maintained at 222 nm wavelength [30] with a data collection rate at 1.0 Hz for the detection

of HNE. CAD detector settings were maintained at 50°C evaporating temperature, data collection rate of 10 Hz and a filter of 3.6 sec. A 30-minute blank run was performed after each set of three repetitions from each sample to ensure optimal column cleaning between samples. This blank ran the same CAD, UV, column and autosampler conditions as above. The flow rate was increased to 0.4 ml min⁻¹ and a 2 µl injection of blank mobile phase was carried out. During this period, mobile phase gradient followed the same pattern as above but was proportionally compressed into 30 minutes. Chromatography control and integration of chromatograms was completed using Chromeleon (ver 7.2) software (Thermo Scientific).

MS conditions for triglyceride identification:

Identification of selected triglycerides from each IVLE was performed by collection of fragments from the HPLC effluent at point of entry to the CAD at time points corresponding to the 5 main peaks in Intralipid® and the 9 main peaks in SMOFlipid®. These main peaks were selected due to the objective of monitoring the changes in triglyceride levels that occur during storage before delivery to the patient, providing an overview of the lipid changes occurring during storage. Therefore, not all peaks in each chromatogram were identified, just the main primary peaks (5 in Intralipid® and 9 in SMOFlipid®). These collected fragments were then analysed by MS, performed on an Agilent 1100 series autosampler and a Bruker MicroTOF ESI-MS. The mass spectrometer was operated in positive ionisation mode with a full scan mode from 0 to 2000 Da. An isocratic mobile phase consisted of MeOH (0.1 % formic acid): water 90:10 at a flow rate of 1ml min⁻¹. Source parameters were as follows: capillary voltage 4.5 kV, end plate offset -500 V, nebuliser pressure (N₂) 0.4 Bar, dry gas (N₂) 4 L min⁻¹, dry heater 200°C. Sample injection size was 30 µl to 60 µl dependant on response. Analysis of MS data was carried on Hystar post acquisition (Bruker Daltonics) software with base peak chromatograms for each sample created from 0 to 1000 Da.

HPLC-UV-CAD method validation:

HNE was quantified by creation of standard curves prepared from a stock solution of HNE standard (10 mg ml⁻¹) in ethanol. This stock solution was further diluted with ethanol to create a 1 mg ml⁻¹ solution. Aliquots of 64, 48, 32, 16 and 8 µl of this stock solution were made up to 2 ml with Intralipid 20 % creating a set of standards for calibration at 204.83, 153.62, 102.51, 51.21 and 25.60 µM HNE respectively. These standard solutions were then subjected to HPLC and calibration curves created to assess linearity. Calibration of selected triglycerides was carried out using Intralipid® 20 % and SMOFlipid® 20 % as internal standards of specific triglycerides are unsuitable due to the emulsion nature of the IVLE's. Using individual standards of each triglyceride to create calibration curves was not employed as elution times of triglycerides not formulated within emulsions will be different to those as seen when analysing the selected IVLE's when using the above assay conditions. Therefore, standard solutions of each lipid emulsion were created by adding volumes of each IVLE to water to obtain 12.5, 25, 50, 75 and 100 % concentrations for each emulsion. Again, these were then subjected to the HPLC conditions detailed above and calibration curves for each triglyceride peak were created. Selectivity, intra-day and inter-day variability and precision was determined for triglycerides through separate sampling of 1 ml of each IVLE and using the stock solution of HNE in Intralipid® and SMOFlipid® as detailed above. Precision was expressed as a percentage of relative SD (RSD) and tested both inter and intra-day by testing standard samples over separate runs on the same day separated by blank runs and on two separate days on different weeks. All samples were freshly prepared on each required day and stored at 2-8°C before analysis.

Results

Optimisation of HPLC-UV-CAD method:

We have presented what is to our knowledge a novel method for the simultaneous analysis of triglycerides within IVLE's and the detection of HNE. Example chromatograms for both Intralipid® 20% and SMOFlipid® 20% show sufficient resolution for quantification (Figs 1 and 2). Intralipid® spiked with 16 µg HNE standard (Fig 3) shows the importance of the initial gradient elution in producing effective separation of HNE from triglyceride peaks. Figures 4 and 5 show Intralipid® 20% and SMOFlipid® 20 % in varying states of degradation, clearly showing the production of HNE in comparison with new fresh samples and samples spiked with HNE standard. Method development and optimisation was carried out initially separately for HNE and triglycerides before placing the UV and CAD detectors in-line with each other creating the final assay conditions. Temperature optimisation of the column was found to be a key governing factor on creating sufficient resolution. Flow rate, CAD conditions and UV data collection rate were all optimised to achieve maximal resolution.

Mass spectrometry for triglyceride analysis:

MS was employed to identify the selected peaks in Intralipid® (Fig 1) and SMOFlipid® (Fig 2). Due to the emulsion formulations being analysed, internal addition of standards for each triglyceride could not be employed for assay validation and peak identification. Therefore, to circumvent this issue fragments of HPLC effluent were collected corresponding to each selected peak for identification. The CAD detector is destructive in its analysis therefore fragments were collected at the entry point to the detector using time points corresponding to each selected peak of interest and subjected to analysis by MS. Data collected from MS is presented in tables 2 and 3 for Intralipid® 20% and SMOFlipid® 20% and analysed using RCM's lipid data [31] and Hystar (Bruker) post processing software. Putative peak identification with the likely fatty acid composition of each triglyceride was carried out. Whilst the sensitivity of the MS used didn't allow runs to distinguish between triglycerides with the same m/z ratio, we can predict the TAG

responsible for each peak analysed. Li et al. [32] shows the analysis of TAGs present within soy bean oil by percentage occurrence. This combined with the prevalence of the individual fatty acids as recorded in each IVLE's Summary of Product Characteristics (SPC) shown in table 1 was used to assign specific TAGs to each selected peak in Intralipid® 20% and peaks 5 to 9 of SMOFlipid® 20%. Peaks 1 to 4 of SMOFlipid® 20% were identified as medium chain saturated fatty acids of C8 to C10 in length.

Method validation for HNE detection:

HNE calibration was performed as described with linear regression analysis giving a R^2 of 0.998 in a concentration range from 25 to 204 μM . The LLOD and LLOQ detailed as the concentration producing signal to noise ratio of 3 and 10 respectively was 3.523 μM and 11.743 μM obtained from injection of standard solutions. Precision was determined as detailed in table 4 and show high levels of precision for HNE over both low (25 μM) and high (204 μM) both intra and inter-day. At all concentrations accuracy fell within acceptable ranges (80 to 120%)[33]. Considered collectively the above data show a sensitive, accurate and precise method for the detection of HNE within IVLE's.

Method validation for IVLE's Intralipid® 20% and SMOFlipid® 20%:

SPC data for both IVLE's provides ranges of concentrations for each fatty acid present. This prevents the individual concentrations of specific triglycerides from being calculated due to the variability of fatty acid concentrations in each IVLE. Individual triglyceride standards cannot be used as internal standards due to the formulation properties of the emulsion. Therefore, to overcome these issues concentrations of 12.5, 25, 50, 75 and 100% of each IVLE diluted with water were created to enable calibration curves to be formed. Triglyceride detection was performed using a CAD detector which typically gives a non-linear response when used over a wide concentration range[34,35], therefore calibration curves for each selected peak were plotted using a second order polynomial function with results for all peaks both in SMOFlipid® and Intralipid® showing good correlation ($R^2 > 0.99$). Precision was calculated for Intralipid® and SMOFlipid® peaks both inter ($n = 3$) and intra-day ($n = 6$) with results yielding a maximum of 4.5 % RSD

(intra-day) and 5.2 % RSD (inter-day) for Intralipid® and 5.0 % RSD (intra-day) and 4.7 % RSD (inter-day) for SMOFlipid®. In combination as detailed in table 5, this data demonstrates a precise and repeatable assay to quantify the selected triglycerides within these IVLE's.

Discussion

This study presents a novel HPLC method utilising in line UV and CAD detectors to monitor triglycerides within Intralipid® and SMOFlipid® IVLE's whilst simultaneously quantifying HNE produced as a result of lipid peroxidation. This aims to address the lack of chemical testing that occurs in such IVLE's during storage, prior to delivery to the patient, by enabling TAG levels to be monitored and HNE levels to be quantified. The method developed has a total chromatographic time of 90 minutes. Whilst this is relatively long in comparison to the time required to employ less accurate assays (FOX) [36], the assay is designed to be used as a stability indicating method, not as a day to day testing method, therefore run time isn't of vital importance and the extended time allows for higher concentrations of IPA to be employed during mobile phase gradient. As IPA is relatively viscous at the low temperatures that the column is held at, the long run time and low flow rate counterbalance this. When employing non-aqueous reversed phase (NARP) chromatography, the difference in polarities of each phase and subsequently the strength assigned to each phase is the governing factor over the phases' ability to elute hydrophobic TAGs from the C30 column [37–39]. In the mobile phase mixture used, IPA constitutes the 'strong' eluting solvent. Initially the gradient employs a high concentration (60 %) of ACN, optimised to effectively separate HNE from both early eluting TAGS of low carbon number and other short chain aldehydic products (fig 3). The isocratic hold of 60 % IPA/40 % ACN forms the main body of the assay from 20 to 70 minutes, creating optimal conditions for TAG elution and effective separations and resolutions. With regards to the order of elution of the TAGs in both Intralipid® and SMOFlipid® all identified TAGs, except the medium chain saturated TAGs in SMOFlipid®, followed the general formula $PN = CN - 2DB$ (PN partition number, CN Carbon number, DB double bonds) [37]. As NARP is employed in the assay with a C30 column the level of unsaturation will govern the level of interaction each fatty acid on each TAG will have with the stationary phase. Due to the significant difference in chain length of the saturated fatty acids (C8 to C10) found in SMOFlipid® these TAGs elute early in the run following the elution order of increasing carbon chain length.

The use of the CAD allows accurate quantification of TAGs without the need for derivatisation or argention, reducing the sample preparation required and ensuring optimal sample recovery. The CAD has a non-linear response over wide concentration ranges, following a second order polynomial function [40]. Throughout calibration RSD's were monitored for both lipid emulsions the maximum being 5.1%, proving an acceptable level of precision and repeatability.

When considering the validation of the CAD section of the assay for the quantification of TAGs, LLOD and LLOQ cannot be calculated due to the limited information given in each of the IVLE's SPC: amounts are given as a range of concentrations of individual fatty acids. Due to this, exact concentrations of each triglyceride attributed to each selected peak cannot be calculated, preventing LLOD and LLOQ from being calculated as a molarity. It is envisioned that the method presented within the paper would be employed by a researcher to develop their own testing protocol for their required data. Whilst the same batch of lipid would be advantageous due to the variabilities mentioned, in practise the use of a control sample used as a comparison of the same batch for each subject tested allows the method to be used as discussed for multiple different batches under any required conditions. We can however express LLOD and LLOQ as a percentage concentration from a neat standard (100 %) of IVLE. For Intralipid® 20 %, the LLOD and LLOQ for the smallest measured peak was 0.64 % and 2.32 % respectively. SMOFlipid® 20 % produced LLOD and LLOQs of 1.57 % and 5.57 %. For the purposes of the use of the assay, such LLOD's and LLOQ's are sufficient validation as, during storage before delivery to the patient, the amount of TAGs lost will be no more than 50 %. The calculation of peak area for each selected peak cannot be translated into a concentration for TAG peaks within these lipid emulsions, therefore data from the assay can be presented as a percentage loss of TAG from an initial day 0 run.

In conclusion, a novel non-aqueous reversed phase HPLC assay has been established that employs in line UV and CAD detection for the accurate quantification of both triglycerides and the peroxidation product 4-hydroxynonenal within lipid emulsions. The combination of two detectors in line permits the detection of both short chain volatile aldehydic molecules and non-volatile complex triglycerides without the need

for complex sample preparation or derivatisation. The method is precise and reliable and can be employed in the chemical stability testing of a variety of lipid emulsions.

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Tables

Table 1. Fatty acid composition of Intralipid® 20 % and SMOFlipid® 20 % as shown in summary of product characteristics (Fresenius Kabi). All are subsequently formulated into triglycerides forming stable intravenous lipid emulsions.

| Fatty acid | Carbon number: double bond | SMOFlipid® 20 % | Intralipid® 20 % |
|--------------------------|----------------------------|-----------------|------------------|
| Oleic acid | C18:1 | 23-35% | 19-30% |
| Linoleic acid | C18:2 | 14-25% | 44-62% |
| Caprylic acid | C8:0 | 13-24% | - |
| Palmitic acid | C16:0 | 7-12% | 7-14% |
| Capric acid | C10:0 | 5-15% | - |
| Stearic acid | C18:0 | 1.5-4% | 1.4-5.5% |
| α -linolenic acid | C18:3 | 1.5-3.5% | 4-11% |
| EPA | C20:5 | 1-3.5% | - |
| DHA | C22:6 | 1-3.5% | - |

Table 2. Mass spectrometry data for selected Intralipid® 20 % peaks and subsequent triglycerides attributed to each peak.

| Intralipid® Peak number | Mass spectrometry m/z data | Triglyceride attributed |
|-------------------------|----------------------------|-------------------------|
| 1 | 879.74 [M+H] ⁺ | TG (18:2/18:2/18:2) |
| 2 | 881.76 [M+H] ⁺ | TG (18:2/18:2/18:1) |
| 3 | 855.74 [M+H] ⁺ | TG (18:2/18:2/16:0) |
| 4 | 885.78 [M+H] ⁺ | TG (18:1/18:1/18:1) |
| 5 | 857.75 [M+H] ⁺ | TG (18:2/18:1/16:0) |

Table 3. Mass spectrometry data for selected SMOFlipid® 20 % peaks and subsequent triglycerides attributed to each peak.

| SMOFlipid® Peak number | Mass spectrometry m/z data | Triglyceride attributed |
|------------------------|---|-------------------------|
| 1 | 493.35 [M+Na] ⁺ | TG (8:0/8:0/8:0) |
| 2 | 521.37 [M+Na] ⁺ | TG (8:0/8:0/10:0) |
| 3 | 549.40 [M+Na] ⁺ | TG (8:0/10:0/10:0) |
| 4 | 577.43 [M+Na] ⁺ | TG (10:0/10:0/10:0) |
| 5 | 879.75 [M+H] ⁺ 901.73 [M+Na] ⁺ | TG (18:2/18:2/18:2) |
| 6 | 881.76 [M+H] ⁺ 903.75 [M+Na] ⁺ | TG (18:2/18:2/18:1) |
| 7 | 855.74 [M+H] ⁺ | TG (18:2/18:2/16:0) |
| 8 | 855.78 [M+H] ⁺ 907.76 [M+Na] ⁺ | TG (18:1/18:1/18:1) |
| 9 | 857.75 [M+H] ⁺ 879.75 [M+Na] ⁺ | TG (18:2/18:1/16:0) |

Table 4. Assay validation of precision and accuracy for HNE.

| | Precision | | Accuracy |
|--|----------------------|----------------------|----------|
| HNE concentration (μM) | RSD ₁ (%) | RSD ₂ (%) | (%) |
| 25.6 | 7.33 | 1.05 | 117.50 |
| 51.21 | 3.07 | 1.70 | 84.35 |
| 102.51 | 7.19 | 3.76 | 96.90 |
| 153.62 | 5.40 | 4.37 | 90.14 |
| 204.83 | 5.53 | 3.75 | 97.39 |

RSD₁, intra-day precision (n = 3), RSD₂, inter-day precision (n = 6)

Table 5. Assay validation data for Intralipid® 20 % and SMOFlipid® 20 % selected peaks.

| | Peak number | R ² | Precision | |
|------------------|-------------|----------------|------------------|------------------|
| | | | RSD ₁ | RSD ₂ |
| Intralipid® 20 % | 1 | 0.996 | 1.931 | 1.689 |
| | 2 | 0.992 | 1.567 | 3.679 |
| | 3 | 0.997 | 4.534 | 2.079 |
| | 4 | 0.995 | 4.225 | 5.209 |
| | 5 | 0.998 | 3.812 | 0.566 |
| SMOFlipid® 20 % | 1 | 0.997 | 3.264 | 3.740 |
| | 2 | 0.997 | 3.090 | 2.597 |
| | 3 | 0.996 | 2.719 | 2.258 |
| | 4 | 0.996 | 3.750 | 1.614 |
| | 5 | 0.997 | 3.520 | 2.048 |
| | 6 | 0.996 | 3.463 | 2.384 |
| | 7 | 0.999 | 2.598 | 3.544 |
| | 8 | 0.991 | 5.081 | 2.607 |
| | 9 | 0.993 | 4.757 | 4.745 |

RSD₁, inter-day repeatability (n =6), RSD₂, intra-day repeatability (n = 3)

Figures.

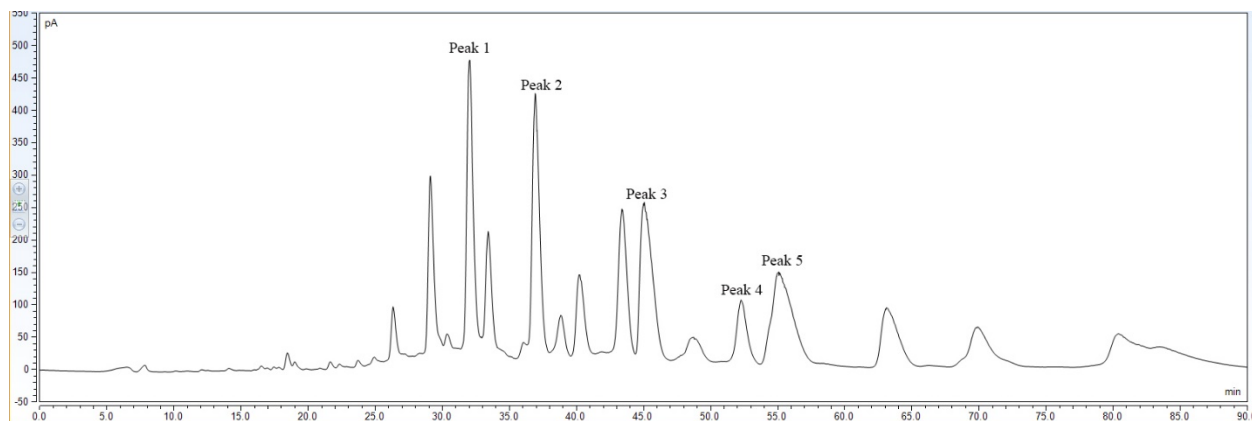


Figure 1. HPLC-CAD chromatogram of Intralipid® 20 % showing selected peaks collected for mass spectrometry and identification.

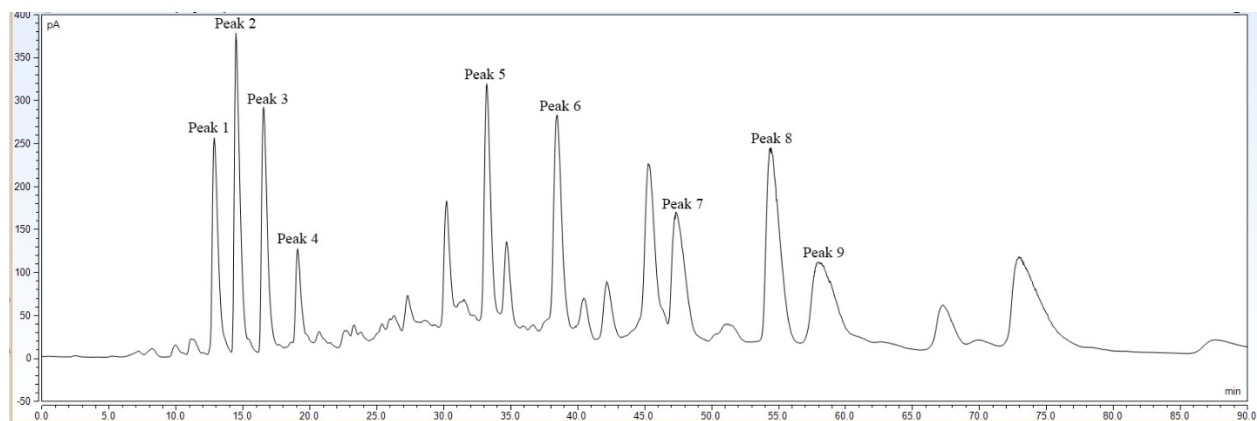


Figure 2. HPLC-CAD chromatogram of SMOFlipid® 20 % showing selected peaks subsequently collected for mass spectrometry identification.

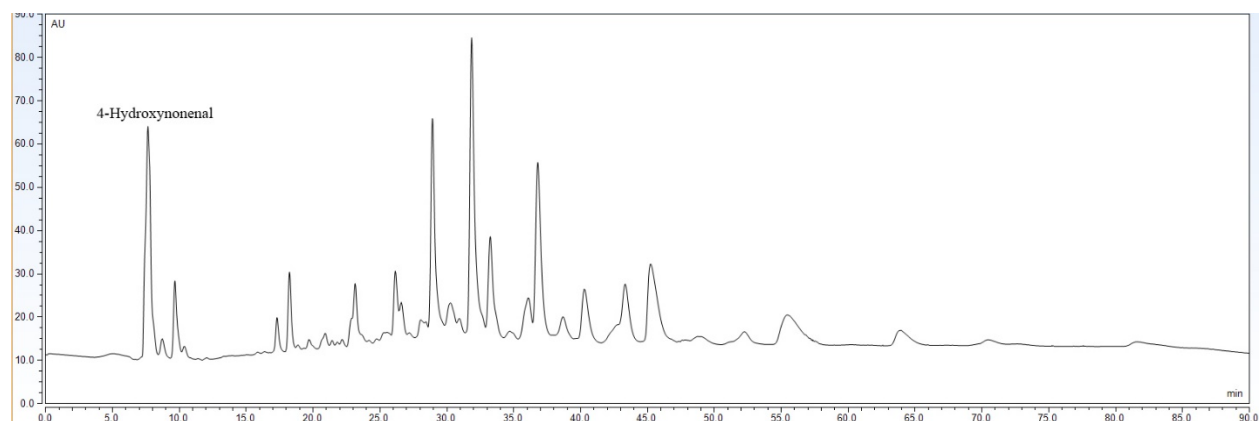


Figure 3. HPLC-UV chromatogram of degraded (84 days at room temperature in a 50ml oxygen permeable syringe) Intralipid® 20 % with additional 16 µg of HNE standard. HNE peak shows clear separation from triglycerides and other peroxidation products that elute early due to their short chain length.

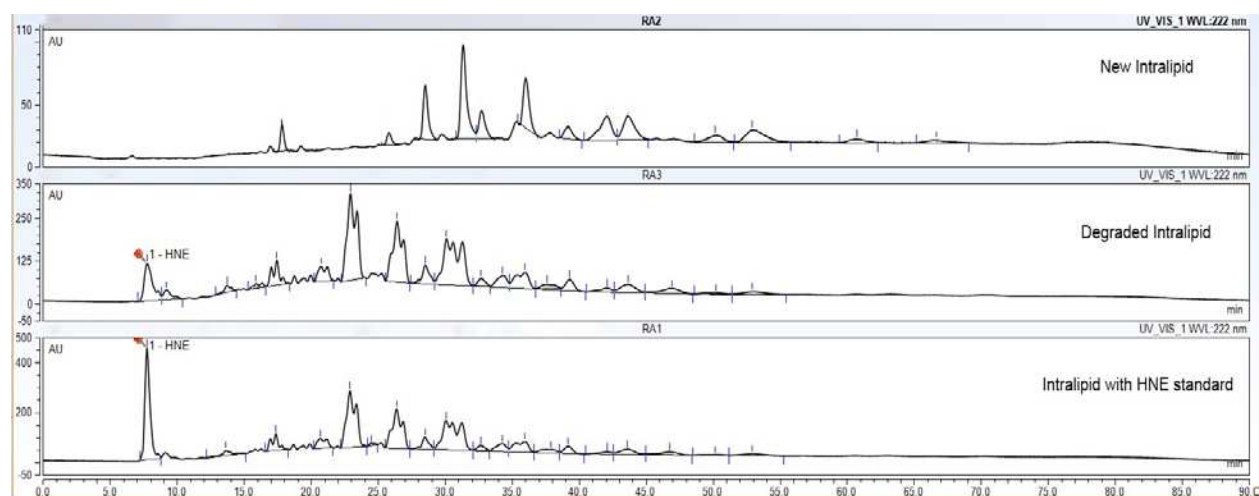


Figure 4: HPLC-UV chromatograms of fresh Intralipid® 20%, degraded Intralipid® 20% and Intralipid with added HNE standard.

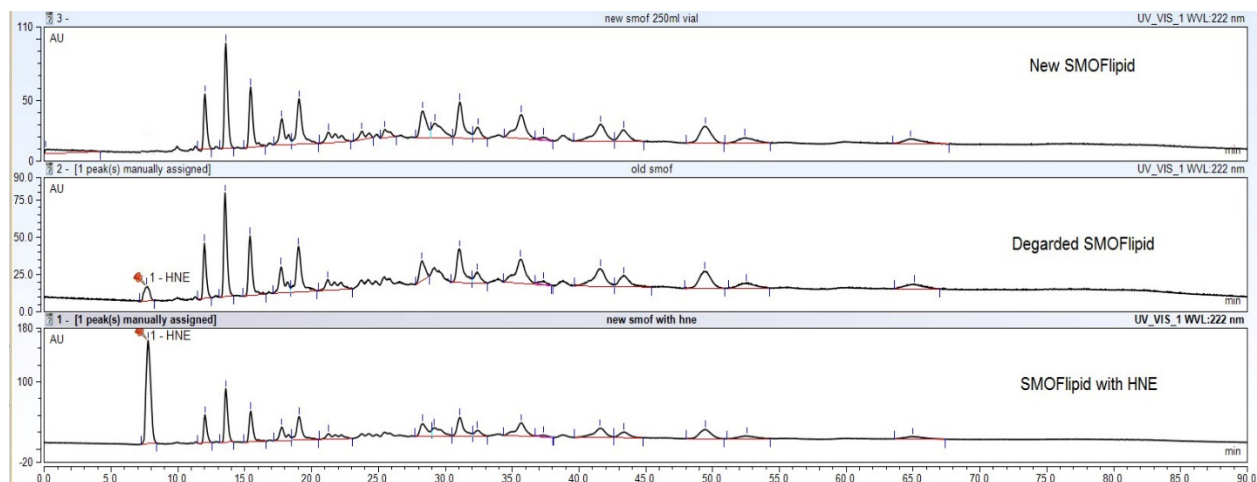


Figure 5: HPLC-UV chromatogram of new SMOFlipid[®] 20%, degraded SMOFlipid[®] 20% and SMOFlipid[®] with added HNE standard.